PF-0459 US
09/002,485
Reference No. 12

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/19, C07K 14/52, 16/24, C12N 15/62, 5/10, A61K 38/19, C12Q 1/68

A1

(11) International Publication Number: **WO 98/44117**

(43) International Publication Date: 8 October 1998 (08.10.98)

(21) International Application Number: PCT/US98/06115

(22) International Filing Date: 27 March 1998 (27.03.98)

(30) Priority Data:

60/042,862	28 March 1997 (28.03.97)	US
60/046,083	9 May 1997 (09.05.97)	US

(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventor: SHEPPARD, Paul, O.; 20717 N.E. 2nd Street, Redmond, WA 98053 (US).

(74) Agent: LINGENFELTER, Susan, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN CHEMOKINE ZSIG-35

(57) Abstract

Novel ligand polypeptides, polynucleotides encoding the novel ligand polypeptides, and related compositions and methods are disclosed. The novel ligand polypeptide is a member of the chemokine β subfamily. The polypeptides may be used within methods relating to regulation of acute and chronic inflammatory disease conditions, lymphocyte migration and ischemia/reperfusion injury.

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                1        10        20        30        40        50
MCPI_RAT        -MQVSVTLLGLLFTVAACSIHVLSQPDAVNA----------------------P

                1        10        20        30        40        50
MMU49513_1      MKPFHTALSFLILTTALGIWAQITHATETKEVQSSLKAQQGLEIEMFHMGFQDS

                1        10        20
zsig35          MNLWLLLACLVAGFLG AWAPA-------------------------VHTQGVP


                    60        70        80        90        100
MCPI_RAT        LTCCYSFTGKMIPMSRLENYKRI---TSSRCPKEAVVFVTKLK-REICADPNKE

                    60        70        80        90        100
MMU49513_1      SDCCLSYNSR-IQCSRFIGYFP----TSGGCTRPGIIFISKRG-FQVCANPSDR

                  30        40        50        60        70        80
zsig35          EDCCLAYHYPIGWAVLRRAWTYRIQEVSGSCNLPAAIFYLPKRHRKVCGNPKSR


                 110       120       130       140       150
MCPI_RAT        WVQKYIRKLDQNQVRSETTVFYKIASTLRTSAPLNVNLTHKSEANASTLFST

                      110       120
MMU49513_1      RVQRCIERLEKNSQPRTYKQ

                        90        100       110       120       130
zsig35          EVQRAMKLLDARNKVFAKLRHNTQTFQAGPHAVKKLSSGNSKLSSSKFSNPI


                          170
MCPI_RAT        TTSSTSVEVTSMTEN

                      140       150
zsig35          SSSKRNVSLLISANSGL
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DESCRIPTION

HUMAN CHEMOKINE ZSIG-35

BACKGROUND OF THE INVENTION

Chemotactic cytokines were originally identified in supernatants of stimulated leukocyte cell cultures and were initially characterized through their chemotactic effects on a variety of leukocytes. Subsequent isolation of several chemotactic cytokines has led to the discovery of a family of structurally and functionally homologous molecules now known as "chemokines" (see Schnell, The Cytokine Handbook, Academic Press, 419-60, 1994). *In vivo*, chemokines have pro-inflammatory, pyrogenic, chemokinetic, myelosuppressive or hematopoietic effects; primarily, chemokines regulate inflammatory and immunoregulatory processes through their selective recruitment and activation of leukocytes. Chemokines are 8 to 16 kDa soluble basic proteins that are produced and released by a variety of cell types during an acute inflammatory response to injury, allergens, or microbial and viral infections. Moreover, chemokine involvement is apparent in some chronic inflammatory states such as arthritis, asthma, and eczema.

The chemokine family contains over 20 members sharing 20-70% amino acid sequence homology. Currently, most isolated chemokines are of human origin, with a few known orthologues reported. Chemokines are mainly divided into two subclasses (alpha (α) and beta (β)) based on a four-cysteine motif contained within their protein structure. The first pair of cysteines are either separated by an intervening residue (the α subfamily, or "C-X-C" chemokines) or are adjacent (the β subfamily, or

"C-C" chemokines). In general, the C-X-C chemokines are involved in neutrophil recruitment and activation and are implicated in acute inflammatory diseases. The C-C chemokines exert their effect on other leukocyte populations such as, monocytes, T cells, eosinophils and basophils and are implicated in chronic inflammatory conditions. However, this functional distinction between the families is not exclusive; for example, the C-X-C chemokine, platelet factor 4 (PF4), induces both neutrophil and monocyte migration. Lymphotactin is a prototype of a third class of chemokines ("C" chemokines) which contain only two of the four cysteines. Further investigation has elucidated other common characteristics of each family.

Most known members of the C-X-C family of chemokines are found clustered on human chromosome 4 between q12 and q21. The well known members of this family are PF4 and interleukin 8 (IL-8). The most extensively studied chemokine, PF4, released from blood platelets, is thought to play a role in inflammation and wound healing. PF4 may provide a model for linking the physiologically coordinated processes of thrombosis, inflammation and wound healing. IL-8, which attracts neutrophils, may influence neutrophil-mediated damage during inflammation. Moreover, IL-8 is implicated in other acute immune reactions (e.g., response to bacterial endotoxins). In fact, anti-IL-8 antibodies prevent the migration of neutrophils to injured lung tissue and protect it from IL-8-induced lung injury *in vivo*, proving a causal role of locally-produced IL-8 in reperfusion injury in a rabbit lung model (Sekido et al., Nature 365:654-57, 1993).

The C-C family of chemokines appears to be clustered on human chromosome 17 between q11 and q21. Thus far, all have a three exon, two intron genomic arrangement. The biological activities of the C-C group

appear more diverse than those of the C-X-C chemokines, indicating that the C-C chemokines may serve as links between monocytes, lymphocytes, eosinophils and basophils in immune and inflammatory responses. Some of the well known members of this family are macrophage inflammatory protein (MIP-1), monocyte chemotactic protein (MCP-1), RANTES, and eotaxin. Unlike nearly all other chemokines, MIP-1 is an acidic protein. It is expressed primarily in mitogen-stimulated T-cells, B-cells and monocytes. MIP-1 has been shown to have hematopoietic effects and pyrogenic effects. Interestingly, MIP-1's fever-inducing properties are independent of the prostaglandin pathway since they are unaffected by aspirin or ibuprofen (which block the cyclooxygenase pathway). Studies show that homozygous MIP-1α null mutant mice are resistant to Coxsackievirus-induced myocarditis, and exhibit reduced pneumonitis and delayed viral clearance during influenza virus infection (Cook et al., Science 269:1583-85, 1995). Thus, murine MIP-1α is required for inflammatory response to viral infection *in vivo*.

Although most C-C chemokines induce a spectrum of biological effects, eotaxin acts very specifically. Eotaxin exclusively attracts eosinophils both *in vivo* and *in vitro*, with no effects on neutrophils or monocytes. Eosinophils are recruited in allergic reactions leading to eczema and asthma as well as in response to certain parasites. Human eotaxin is expressed at high levels in the small intestine and colon, and appears to have a specific interaction with only one member of the chemokine receptor family (CC CKR3) that is selectively expressed on human eosinophils (Kitaura et al., J. Biol. Chem. 271:7725-30, 1996). This evidence of narrow specificity for a chemokine, eotaxin, implies the existence of undiscovered chemokines with specific biological activities.

Although chemokines exhibit a wide variety of biological activities that affect inflammatory cells, they appear to share interaction with only a few receptors. The specific effects of chemokines are mediated by a family of 7-transmembrane domain G-protein coupled receptors. How chemokines exhibit specific and varied effects via their receptors is generally unknown. For instance, MIP-1, MCP-1 and RANTES all bind C-C chemokine receptor 1 (CC CKR1), but how the biological specificity is imparted by each is undefined. Several groups have shown that chemokine receptors react to different combinations of chemokines in different ways. For example, Ca^{++} signaling desensitization studies suggest an array of shared and specific receptors; moreover, the desensitization pattern depends on the order of chemokine involvement and the cell type to which they are exposed. Moreover, differences in binding affinities and signaling activities with shared receptors are exhibited. *In vivo*, it is likely that both chemokine and specific chemokine receptor expression are regulated temporally and spatially.

Because chemokines exhibit diverse biological activities, they are believed to be involved with several pathological conditions. Chemokines are implicated in inflammation, ischemia and reperfusion injury, wound healing, allergies, bacterial and viral pathogenesis. Moreover, chemokines may be involved in chronic diseases such as arthritis, asthma and atherosclerosis (migration of monocytes and arterial damage). For example, MCP-1 mRNA is detected in a variety of conditions where monocytes predominate in the pathology; moreover, high levels of chemokines have been detected in certain disease states. Many tumor cell lines and some primary tumors produce chemokines. Whether chemokines are active in tumor biology is unknown, but the migratory and immune effects of these molecules may implicate a role in tumor

regression or growth. Although there are correlations between chemokines and disease, the causal relationship and roles are not well defined. DARC, a promiscuous chemokine receptor is also a receptor for the malarial parasite (Horuk et al., Science 261:1182-84, 1993) and the chemokine receptor CCCK5 is involved with HIV. Animal studies in which antibodies are used to neutralize the activity of individual members of the chemokine family confirm that these mediators contribute to the development of both acute and chronic inflammatory disease conditions. Because of their association with certain disease states, lymphocyte migration, immune and inflammatory regulation, chemokines and their inhibitors may prove useful as therapeutics.

The demonstrated *in vitro* and *in vivo* activities of these chemokine family members illustrate the enormous clinical potential of, and need for, other chemokine ligands, chemokine receptors and chemokine agonists and antagonists. Therapeutics that target chemokines directly or enhance the body's mechanisms for controlling their actions may prove to be reasonable approaches for treatment of a number of disease states. The present invention addresses this need by providing a novel human chemokine and related compositions and methods.

SUMMARY OF THE INVENTION

Within one aspect the invention provides an isolated polypeptide comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, the polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2, wherein the polypeptide is a chemokine. Within one embodiment the polypeptide is at least 90% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, the polypeptide comprising cysteine residues at positions

corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2. Within another embodiment the polypeptide comprises the sequence of amino acid residues from residue 16-150 as shown in SEQ ID NO:2. Within another embodiment the polypeptide comprises the sequence of amino acid residues from residue 21-150 as shown in SEQ ID NO:2. Within yet another embodiment the polypeptide comprises the sequence of amino acid residues from residue 1-150 as shown in SEQ ID NO:2. Within another embodiment the polypeptide further comprises an affinity tag which is polyhistidine, protein A, glutathione S transferase, substance P, FLAG, Glu-Glu or an immunoglobulin heavy chain constant region. Within a related embodiment is provided a proteolytic cleavage site between the sequence of amino acid residues and the affinity tag.

Within another aspect of the invention is provided an isolated polynucleotide encoding a polypeptide as described above. Within one embodiment the polynucleotide comprises a sequence of nucleotides as shown in SEQ ID NO:14 from nucleotide 1 through nucleotide 450. Within another embodiment the polynucleotide comprises a sequence of nucleotides as shown in SEQ ID NO:1 from nucleotide 162 through nucleotide 551. Within another embodiment the polynucleotide is from 390 base pairs to 450 base pairs in length. Within yet another embodiment the polynucleotide is DNA.

Within another aspect the invention provides an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as described above; and a transcription terminator. Within one embodiment the expression vector further comprises a secretory signal sequence operably linked to said DNA segment. Within a related embodiment the secretory signal sequence comprises amino acid residues 1-20 of SEQ ID NO:2. Within another embodiment is provided a cultured cell into which has been introduced

an expression vector as described above, wherein the cell expresses the polypeptide encoded by the DNA segment. Within another embodiment is provided a method of producing a protein comprising: culturing a cell into which has been introduced an expression vector as described above whereby the cell expresses the protein encoded by the DNA segment; and recovering the expressed protein.

Within other aspects of the invention are provided an antibody that specifically binds to an epitope of a polypeptide as described above. A binding protein that specifically binds to an epitope of a polypeptide as described above. A pharmaceutical composition comprising a polypeptide as described above and a pharmaceutically acceptable carrier.

Within another aspect is provided an oligonucleotide probe or primer comprising 14 contiguous nucleotides of a polynucleotide of SEQ ID NO:14 or a sequence complementary to SEQ ID NO:14. Within one embodiment the oligonucleotide probe is labeled to provide a detectable signal.

Within yet another aspect of the invention is provided a method for detecting a genetic abnormality in a patient, comprising: obtaining a genetic sample from a patient; incubating the genetic sample with a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; comparing the first reaction product to a control reaction product, wherein a difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawing.

BRIEF DESCRIPTION OF THE DRAWING

The Figure shows a comparison of the deduced amino acid sequence of ZSIG-35 (SEQ ID NO:2) with the deduced amino acid sequence of mouse MIP-1γ (SEQ ID NO:10) and rat MCPI (SEQ ID NO:11). Deduced signal sequence and mature protein regions are noted for ZSIG-35. The cysteine residues are indicated in bold typeface.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter:

Affinity tag: is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. USA 82:7952-4, 1985), substance P, Flag™ peptide (Hopp et al., Biotechnology 6:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

Allelic variant : Any of two or more alternative forms of a gene occupying the same chromosomal locus.

Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (i.e., no change in the encoded polypeptide), or may encode polypeptides having altered amino acid sequence. The term "allelic variant" is also used herein to denote a protein encoded by an allelic variant of a gene. Also included are the same protein from the same species which differs from a reference amino acid sequence due to allelic variation. Allelic variation refers to naturally occurring differences among individuals in genes encoding a given protein.

Complements of a polynucleotide molecule: is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

Degenerate nucleotide sequence: denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

Expression vector: A DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and optionally one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

Isolated: When applied to a polynucleotide molecule, the term "isolated" indicates that the molecule

is removed from its natural genetic milieu, and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, and may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

Isolated polypeptide or protein: is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

Operably linked: As applied to nucleotide segments, the term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

Ortholog: denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

Polynucleotide: denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

Polypeptide: is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

Prompter: is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

Protein: is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins

are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

Receptor: A cell-associated protein, or a polypeptide subunit of such protein, that binds to a bioactive molecule (the "ligand") and mediates the effect of the ligand on the cell. Binding of ligand to receptor results in a change in the receptor (and, in some cases, receptor multimerization, i.e., association of identical or different receptor subunits) that causes interactions between the effector domain(s) of the receptor and other molecule(s) in the cell. These interactions in turn lead to alterations in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, cell proliferation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

Secretory signal sequence: A DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein

encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

The present invention is based in part upon the discovery of a novel DNA sequence (SEQ ID NO:1) and corresponding polypeptide sequence (SEQ ID NO:2) which have homology to members of the β chemokine family, in particular, to mouse MIP-1γ. This ligand has been designated ZSIG-35.

Novel ZSIG-35 ligand-encoding polynucleotides and polypeptides of the present invention were initially identified by querying an expressed sequence tag (EST) database. Using this information, a novel 1029 bp human cDNA fragment (SEQ ID NO:1) was obtained. Sequence analysis of a deduced amino acid sequence of ZSIG-35, as represented by SEQ ID NO:2, indicates the presence of a 15 amino acid residue signal sequence from Met, amino acid residue 1 to Gly, amino acid residue 15, and a 145 amino acid residue mature polypeptide from Ala, amino acid residue 16 to Leu, amino acid residue 150. Alternatively, the signal sequence cleavage site may follow Ala, amino acid residue 20 (SEQ ID NO:2) of the encoded ZSIG-35 polypeptide. Those skilled in the art will recognize that these domain boundaries are approximate, and are based on alignments with known proteins and predictions of protein folding. A comparison of the ZSIG-35 deduced amino acid sequence (as represented in SEQ ID NO:2) with the deduced amino acid sequence of mouse MIP-1γ (SEQ ID NO:10) and rat MCPI (SEQ ID NO:11) is shown in the Figure. ZSIG-35 shares 35.8% amino acid identity with mouse MIP-1γ and 20.7% amino acid identity with rat MCPI.

One characteristic of the chemokine family is a motif of four conserved cysteine residues in the mature protein. In the β chemokine family, the first two of these cysteines are adjacent. ZSIG-35 shares this β chemokine four-cysteine motif; the first two cysteines are adjacent at residues 30 and 31, and the other two cysteine residues are at residues 58 and 75 of the deduced amino acid sequence represented in SEQ ID NO:2. Mouse MIP-1γ contains two additional cysteines which are not shared by other members of the β chemokine family or ZSIG-35. Also, unlike nearly all other chemokines, MIP-1 α and β are acidic proteins (pI=4.5). The predicted pI of the ZSIG-35 polypeptide represented by SEQ ID NO:2 is 11.40. These features indicate that the polypeptide encoded by the DNA sequence as represented by SEQ ID NO:1 is a member of the β chemokine family.

Northern blot analysis of various human tissues was performed using a 30 bp DNA probe (SEQ ID NO:9). A predominant transcript of ~1.0 kb was found in thymus and small intestine. Northern blot analysis of mouse MIP-1γ showed abundant expression liver, lung and thymus (Poltorak et al., J. Inflamm. 45:207-19, 1995). Human eotaxin is expressed in small intestine, colon and heart (Ponath et al., J. Clin. Invest. 97:604-12, 1996).

Chromosomal localization of ZSIG-35 to 19p13.3 was determined using radiation hybrid chimeras. Other members of the C-C chemokine family have mapped to human chromosome 17 between q11 and q21.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the ZSIG-35 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:14 is a degenerate DNA sequence that encompasses all DNAs that encode the ZSIG-35 polypeptide

of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:14 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U (uracil) for T (thymine). Thus, ZSIG-35 polypeptide-encoding polynucleotides comprising nucleotide 1 to nucleotide 450 of SEQ ID NO:14 and their RNA equivalents are contemplated by the present invention. Table 1 sets forth the one-letter codes used within SEQ ID NO:14 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C (cytosine) or T, and its complement R denotes A (adenine) or G (guanine), A being complementary to T, and G being complementary to C.

TABLE 1

Nucleotide	Resolution	Nucleotide	Complement
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NO:14, encompassing all possible codons for a given amino acid, are set forth in Table 2.

TABLE 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, et al., Nuc. Acids Res. 8:1893-912, 1980; Haas, et al. Curr. Biol. 6:315-24, 1996; Wain-Hobson, et al., Gene 13:355-64, 1981; Grosjean and Fiers, Gene 18:199-209, 1982; Holm, Nuc. Acids Res. 14:3075-87, 1986; Ikemura, J. Mol. Biol. 158:573-97, 1982. As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential

codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:14 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

Within preferred embodiments of the invention, isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, or to a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is up to about 0.03 M at pH 7 and the temperature is at least about 60°C. As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. It is generally preferred to isolate RNA from leukocytes, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. Polynucleotides encoding ZSIG-35 polypeptides are then identified and isolated by, for example, hybridization or PCR.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO:1 represents a single allele of human ZSIG-35 and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the ZSIG-35 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention further provides counterpart ligands and polynucleotides from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are ZSIG-35 ligand polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate receptors. Orthologs of human ZSIG-35 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the ligand. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A ligand-

encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequence. A cDNA can also be cloned using the polymerase chain reaction (PCR) (Mullis, U.S. Patent No. 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the ligand. Similar techniques can also be applied to the isolation of genomic clones.

The present invention also provides isolated chemokine ligand polypeptides that are substantially homologous to the ligand polypeptide of SEQ ID NO:2 and its species orthologs. By "isolated" is meant a protein or polypeptide that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein or polypeptide is substantially free of other proteins or polypeptides, particularly other proteins or polypeptides of animal origin. It is preferred to provide the proteins or polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. The term "substantially homologous" is used herein to denote proteins or polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO:2 or its orthologs. Such proteins or polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2 or its orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap

opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the ZSIG-35 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 4

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine

Small:	glycine
	alanine
	serine
	threonine
	methionine

The present invention further provides a variety of other polypeptide fusions [and related multimeric proteins comprising one or more polypeptide fusions]. For example, a ZSIG-35 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-ZSOIG-35 polypeptide fusions can be expressed in genetically engineered cells [to produce a variety of multimeric ZSIG-35 analogs]. Auxiliary domains can be fused to ZSIG-35 polypeptides to target them to specific cells, tissues, or macromolecules (e.g., collagen). For example, a ZSIG-35 polypeptide or protein could be targeted to a predetermined cell type by fusing a ZSIG-35 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A ZSIG-35 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., Connective Tissue Research 34:1-9, 1996.

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine,

homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for ZSIG-35 amino acid residues.

Essential amino acids in the ligand polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-85, 1989; Bass et al., Proc. Natl. Acad. Sci. USA 88: 4498-502, 1991; Bajorath et al., Biochemistry 34: 1833-44, 1995; and Bajorath et al., Biochemistry 34: 9844-92, 1995). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., receptor binding and signal transduction) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity; in conjunction with mutation of putative contact site amino acids. See, for example, Jones et al., Nature 388:225-8, 1989; de Vos et al., Science 255:306-12, 1992; Eck et al., J. Biol. Chem. 267:2119-22, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992 and Banner et al., Cell 73:431-5, 1993. The identities of essential amino acids can also be inferred from analysis of homologies with related chemokine ligands.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-56, 1989). Briefly, these

authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-37, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Variants of the disclosed ZSIG-35 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, Nature 370:389-91, 1994, Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-51, 1994 and WIPO Publication WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect activity of cloned, mutagenized ligands. Mutagenized DNA molecules that encode active ligands or portions thereof (e.g., receptor-binding fragments) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

The ligand polypeptides of the present invention, including full-length ligand polypeptides, ligand fragments (e.g., receptor-binding fragments), and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc.

In general, a DNA sequence encoding a ZSIG-35 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a ZSIG-35 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of ZSIG-35, or

may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the ZSIG-35 DNA sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the polypeptides of the present invention is used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. A secretory signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid residues 1-15 of SEQ ID NO:2 is be operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics

7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-45, 1982), DEAE-dextran mediated transfection (Ausubel et al., ibid), and liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g., CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as

"amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from Autographa californica nuclear polyhedrosis virus (AcNPV). DNA encoding the ZSIG-35 polypeptide is inserted into the baculoviral genome in place of the AcNPV polyhedrin gene coding sequence by one of two methods. The first is the traditional method of homologous DNA recombination between wild-type AcNPV and a transfer vector containing the ZSIG-35 flanked by AcNPV sequences. Suitable insect cells, e.g. SF9 cells, are infected with wild-type AcNPV and transfected with a transfer vector comprising a ZSIG-35 polynucleotide operably linked to an AcNPV polyhedrin gene promoter, terminator, and flanking

sequences. See, King and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and, Richardson, C. D., Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. Natural recombination within an insect cell will result in a recombinant baculovirus which contains ZSIG-35 driven by the polyhedrin promoter. Recombinant viral stocks are made by methods commonly used in the art.

The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow et al., J. Virol. 67:4566-79, 1993). This system is sold in the Bac-to-Bac kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the ZSIG-35 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case ZSIG-35. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as P*cor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins and Possee, J. Gen. Virol. 71:971-6, 1990; Bonning et al., J. Gen. Virol. 75:1551-6, 1994; and, Chazenbalk and Rapoport, J. Biol. Chem. 270:1543-9, 1995. In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native ZSIG-35 secretory signal sequences with

secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native ZSIG-35 secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed ZSIG-35 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. 82:7952-4, 1985). Using a technique known in the art, a transfer vector containing ZSIG-35 is transformed into *E. coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses ZSIG-35 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High Five™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent #5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cell0405™ (JRH Biosciences, Lenexa, KS) or Express Five™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately 2-5 x 10^5

cells to a density of 1-2 x 10^6 cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. The recombinant virus-infected cells typically produce the recombinant ZSIG-35 polypeptide at 12-72 hours post-infection and secrete it with varying efficiency into the medium. The culture is usually harvested 48 hours post-infection. Centrifugation is used to separate the cells from the medium (supernatant). The supernatant containing the ZSIG-35 polypeptide is filtered through micropore filters, usually 0.45 μm pore size. Procedures used are generally described in available laboratory manuals (King and Possee, ibid.; O'Reilly et al., ibid.; Richardson, ibid.). Subsequent purification of the ZSIG-35 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae, Pichia pastoris, Pichia methanolica*. Methods for transforming *S. cerevisiae* with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *S. cerevisiae* is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman

et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986; and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the

absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (τ) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., ibid.). When expressing a ZSIG-35 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and

recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and 0.006% L-leucine).

ZSIG-35 polypeptides or fragments thereof may also be prepared through chemical synthesis. ZSIG-35 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

It is preferred to purify the protein to >80% purity, more preferably to >90% purity, even more preferably >95%, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of

infectious and pyrogenic agents. Preferably, a purified protein is substantially free of other proteins, particularly other proteins of animal origin.

Expressed recombinant ZSIG-35 polypeptides (or chimeric ZSIG-35 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art.

Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of specific properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., maltose-binding protein, FLAG tag, Glu-Glu tag, an immunoglobulin domain) may be constructed to facilitate purification.

The invention also provides ZSIG-35 polypeptides with C-terminal extensions. To facilitate purification of the ZSIG-35 polypeptide, a C-terminal extension, such as a poly-histidine tag, substance P, Glu-Glu tag (Grussenmeyer et al., ibid., Flag™ peptide (Hopp et al., ibid.) or another polypeptide or protein for which an antibody or other specific binding agent is available, can be fused to the mature polypeptide.

The activity of molecules of the present invention can be measured using a variety of *in vitro* and

in vivo assays. These assays can also be used to identify cells expressing a ZSIG-35 associated receptor. Competitive binding of ZSIG-35 ligand to receptors on purified normal leukocyte cell populations isolated from human peripheral blood can be done to confirm expression of a receptor. Transient elevation of Ca^{2+} upon chemokine binding to a cell surface receptor can be used to monitor receptor activation. Measurement of intracellular cytosolic free calcium can be done by adding ZSIG-35 to leukocyte cell populations loaded with CA^{2+} indicator dyes such as, Indo-1 or Fura-2 (Kitaura et al., J. Biol. Chem. 271:7725-30, 1996 and Ponath et al., J. Clin. Invest. 97:604-12, 1996). Transendothelial chemotaxis assays (Ponath et al., ibid.) can be used to determine the ability of ZSIG-35 to induce a chemotactic response *in vitro*. This can also be done to identify receptor-bearing cells from leukocyte subpopulations.

To determine if ZSIG-35 is a chemotractant *in vivo,* ZSIG-35 can be given by intradermal or intraperitoneal injection. Characterization of the accumulated leukocytes at the site of injection can be determined using lineage specific cell surface markers and fluorescence immunocytometry or by immunohistochemistry (Jose, J. Exp. Med. 179:881-87, 1994). Release of specific leukocyte cell populations from bone marrow into peripheral blood can also be measured after ZSIG-35 injection.

The role of ZSIG-35 in inflammation and respiratory hypersensitivity can be measured using known animal models. For example, models of allergic inflammation are performed in guinea pigs sensitized with ovalbumin by aerosol challenge. Bronchoaleolar lavage (BAL) fluid containing chemotractant activity from the sensitized animals is then injected intradermally into unsensitized animals and the accumulation of cells, such as eosinophils, at the site of injection is measured (Jose

et al., ibid.). The affects of ZSIG-35 or ZSIG-35 antagonists can be measured to determine their ability to exacerbate or reduce cell infiltration after respiratory inflammation.

A common biological response to cytokines and chemokines is fever. *In vivo* pyrogenicity studies can be done to determine if ZSIG-35 can elicit fever. ZSIG-35 is administered by either systemic or intracerebroventricular routes to mice and core body temperature is measured (Poltorak et al., J. Inflamm. 45:207-19, 1995). An increase in temperature would indicate that the compound is pyrogenic. Measurements can also be taken to determine the increase in ZSIG-35 expression following injury, such as intestinal injury, and subsequent increase in leukocytes at the site of injury. ZSIG-35 can also be tested in models of ischemia/reperfusion injury.

An alternative *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see Becker et al., Meth. Cell Biol. 43:161-89, 1994; and Douglas and Curiel, Science & Medicine 4:44-53, 1997). The adenovirus system offers several advantages: adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with a large number of available vectors containing different promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the

viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (the human 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a secretory signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. Alternatively, adenovirus vector infected 293S cells can be grown in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier et al., Cytotechnol. 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant. Within the infected 293S cell production protocol, non-secreted proteins may also be effectively obtained.

ZSIG-35 ligand polypeptides and soluble ZSIG-35 ligands may be used to identify and characterize receptors

in the chemokine receptor family. Proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column (Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, 195-202). Proteins and peptides can also be radiolabeled (Methods in Enzymol., vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Acad. Press, San Diego, 1990, 721-37) or photoaffinity labeled (Brunner et al., Ann. Rev. Biochem. 62:483-514, 1993 and Fedan et al., Biochem. Pharmacol. 33:1167-80, 1984) and specific cell-surface proteins can be identified. The soluble ligand is useful in studying the distribution of receptors on tissues or specific cell lineages, and to provide insight into receptor/ligand biology.

ZSIG-35 polypeptides can also be used to prepare antibodies that specifically bind to ZSIG-35 epitopes, peptides or polypeptides. Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs, chickens, rabbits, hamsters, guinea pigs, mice, and rats. In addition, antibodies can also be generated from transgenic animals, such as transgenic mice, goats, cows and sheep. The ZSIG-35 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal and elicit an immune response. Suitable antigens would include a contiguous 9 to 150 amino acid fragment thereof. Antibodies generated from this immune response can be isolated and purified as described herein.

The immunogenicity of a ZSIG-35 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of ZSIG-35 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments thereof, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fc fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to ZSIG-35 protein or peptide, and selection of antibody display libraries in

phage or similar vectors (for instance, through use of immobilized or labeled ZSIG-35 protein or peptide).

Antibodies are defined to be specifically binding if they bind to a ZSIG-35 polypeptide with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art (for example, by Scatchard analysis). Cross-reactivity can be determined by methods known in the art, such as by Western Blot analysis. ZSIG-35 antibodies can be "screened against" populations of polypeptides to isolate a population which binds selectively to ZSIG-35. For example, antibodies raised to ZSIG-35 are adsorbed to other polypeptides adhered to insoluble matrix; antibodies which selectively bind ZSIG-35 will flow through the matrix under the proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to the screened polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., Adv. in Immunol. 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J.W. (eds.), Academic Press Ltd., 1996; Benjamin et al., Ann. Rev. Immunol. 2: 67-101, 1984.

Genes encoding polypeptides having potential ZSIG-35 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random

mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the ZSIG-35 sequences disclosed herein to identify proteins which bind to ZSIG-35, "binding proteins".

A variety of assays known to those skilled in the art can be utilized to detect antibodies and binding proteins which specifically bind to ZSIG-35 proteins or peptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, ELISA, dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant ZSIG-35 protein or peptide.

Antibodies and binding proteins to ZSIG-35 may be used for tagging of cells that express human ZSIG-35, for example, to use in a diagnostic assays; for isolating ZSIG-35 by affinity purification; for screening expression libraries; for generating anti-idiotypic antibodies; for detecting circulating levels of ZSIG-35 and as a marker

for underlining pathology or disease; and as neutralizing antibodies or as antagonists to block ZSIG-35 *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to ZSIG-35 or fragments thereof may be used *in vitro* to detect denatured ZSIG-35 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

Antibodies may be made to ZSIG-35 polypeptides containing C-terminal extensions. In particular, antiserum containing polypeptide antibodies to His-tagged soluble ZSIG-35 can be used in analysis of tissue distribution of ZSIG-35 or receptors that bind ZSIG-35 by immunohistochemistry on human or primate tissue.

ZSIG-35 polypeptides can also be used to immunize mice in order to produce monoclonal antibodies to a human ZSIG-35 polypeptide. Monoclonal antibodies to a human ZSIG-35 polypeptide can also be used to mimic ligand/receptor coupling, resulting in activation or inactivation of the ligand/receptor pair. Monoclonal antibodies to ZSIG-35 can be used to determine the distribution, regulation and biological interaction of the ZSIG-35 receptor/ZSIG-35 ligand pair on specific cell lineages. The seven known chemokine receptors have limited expression patterns which are restricted to monocytes, neutrophils, T cells, B cells, basophils, eosinophils, monocytes, macrophages and in one case endothelial cells (Power and Wells, Trends in Pharm. Sci.

17:209-13, 1996). Antibodies to ZSIG-35 can also be used to detect secreted soluble ZSIG-35 in biological samples.

The ZSIG-35 polynucleotides and polypeptides of the present invention may be used as tools for research and for discovery of therapeutic and diagnostic uses of chemokines. Numerous chemokine proteins, polypeptides, antibodies and polynucleotides for use in such methodologies are available for research use from many commercial sources, such as R & D Systems, Minneapolis, MN. ZSIG-35 polypeptides can be used as standards to calibrate *in vitro* chemokine assay systems or as standards within such assay systems. In addition, antibodies to ZSIG-35 polypeptides could be used in assays for neutralization of bioactivity, and as negative controls for a variety of applications, such as ELISA and ELISPOT assays, Western blot, immunohistochemistry, and intracellular staining.

The invention also provides isolated and purified ZSIG-35 polynucleotide probes. Such polynucleotide probes can be ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). DNA can be either complementary DNA (cDNA) or genomic DNA. Polynucleotide probes are single or double-stranded DNA or RNA, generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences and will generally comprise at least 16 nucleotides, more often from 17 nucleotides to 25 or more nucleotides, sometimes 40 to 60 nucleotides, and in some instances a substantial portion or even the entire ZSIG-35 gene or cDNA. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably 15 or more nucleotides, more preferably 20-30 nucleotides. Short polynucleotides can be used when a small region of the gene is targeted for analysis. For gross analysis of genes, a

polynucleotide probe may comprise an entire exon or more. The synthetic oligonucleotides of the present invention have at least 80% identity to a representative ZSIG-35 DNA sequence (SEQ ID NO:1) or its complements. Preferred regions from which to construct probes include the 5' and/or 3' coding sequences, receptor binding regions, signal sequences and the like. Techniques for developing polynucleotide probes and hybridization techniques are known in the art, see for example, Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1991. For use as probes, the molecules can be labeled to provide a detectable signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle and the like, which are commercially available from many sources, such as Molecular Probes, Inc., Eugene, OR, and Amersham Corp., Arlington Heights, IL, using techniques that are well known in the art.

Such probes can also be used in hybridizations to detect the presence or quantify the amount of ZSIG-35 gene or mRNA transcript in a sample. ZSIG-35 polynucleotide probes could be used to hybridize to DNA or RNA targets for diagnostic purposes, using such techniques such as fluorescent *in situ* hybridization (FISH) or immunohistochemistry.

Polynucleotide probes could be used to identify genes encoding ZSIG-35-like proteins. For example, ZSIG-35 polynucleotides can be used as primers and/or templates in PCR reactions to identify other novel members of the chemokine family.

Such probes can also be used to screen libraries for related sequences encoding novel chemokines. Such screening would be carried out under conditions of low stringency which would allow identification of sequences which are substantially homologous, but not requiring complete homology to the probe sequence. Such methods and

conditions are well known in the art, see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989. Such low stringency conditions could include hybridization temperatures less than 42°C, formamide concentrations of less than 50% and moderate to low concentrations of salt. Libraries may be made of genomic DNA or cDNA.

Polynucleotide probes are also useful for Southern, Northern, or slot blots, colony and plaque hybridization and *in situ* hybridization. Mixtures of ZSIG-35 polynucleotide probes can be prepared which would increase sensitivity or the detection of low copy number targets, in screening systems.

In addition, such polynucleotide probes could be used to hybridize to counterpart sequences on individual chromosomes. Chromosomal identification and/or mapping of the ZSIG-35 gene could provide useful information about gene function and disease association. Many mapping techniques are available to one skilled in the art, for example, mapping somatic cell hybrids, and fluorescence *in situ* hybridization (FISH). A preferred method involves PCR to map a gene of interest using the Human/Rodent Somatic Cell Hybrid Mapping Panel Number 2 (National Institute of General Medical Sciences, Cornell Institute of Medical Research, Camden, NJ). The panel consists of DNA isolated from 24 human/rodent somatic cell hybrids, each retaining one specific human chromosome and the parental DNAs. Once the chromosome has been identified, subchromosomal mapping can be done using FISH.

The present invention also provides reagents for use in diagnostic applications. For example, the ZSIG-35 gene, a probe comprising ZSIG-35 DNA or RNA, or a subsequence thereof can be used to determine if the ZSIG-35 gene is present on chromosome 19 or if a mutation has occurred. Detectable chromosomal aberrations at the ZSIG-35 gene locus include, but are not limited to, aneuploidy,

gene copy number changes, insertions, deletions, restriction site changes and rearrangements. These aberrations can occur within the coding sequence, within introns, or within flanking sequences, including upstream promoter and regulatory regions, and may be manifested as physical alterations within a coding sequence or changes in gene expression level.

In general, these diagnostic methods comprise the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with a polynucleotide probe or primer as disclosed above, under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; and (iii) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within the present invention include genomic DNA, cDNA, and RNA. The polynucleotide probe or primer can be RNA or DNA, and will comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, PCR Methods and Applications 1:5-16, 1991), ribonuclease protection assays, and other genetic linkage analysis techniques known in the art (Sambrook et al., ibid.; Ausubel et. al., ibid.; Marian, Chest 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., ibid., ch. 4) comprise the hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient's

genetic sample is incubated with a pair of polynucleotide primers, and the region between the primers is amplified and recovered. Changes in size or amount of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, PCR Methods and Applications 1:34-8, 1991).

ZSIG-35 polypeptides also may be used within diagnostic systems. Chemokines have been detected in a wide variety of tissues and fluid samples, such as those associated with many inflammatory conditions. For example, chemokines have been detected in asthmatic reaction lavage fluid, wound healing site tissue, arteriosclerosis tissue, inflamed gastrointestinal tissue, rheumatoid arthritis synovial fluid and psoriatic scale tissue. Antibodies or other agents that specifically bind to ZSIG-35 may be used to detect the presence of circulating ZSIG-35 polypeptides. Such detection methods are well known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay. Immunohistochemically labeled antibodies can be used to detect ZSIG-35 polypeptides in tissue samples. ZSIG-35 levels can also be monitored by such methods as RT-PCR, where ZSIG-35 mRNA can be detected and quantified. Such methods could be used as diagnostic tools to monitor and quantify receptor or ligand polypeptide levels. The information derived from such detection methods would provide insight into the significance of ZSIG-35 ligand polypeptides in various diseases, and would serve as an early and accurate diagnostic marker for diseases for which altered levels of ZSIG-35 are significant. In particular, ZSIG-35 may be an indicator for treatment in early stages of disease development before clinical symptoms manifest, such as in the case of tumor development. Altered levels of ZSIG-35 ligand polypeptides may be indicative of pathological

conditions, including infections, cancer, myelopoietic disorders, autoimmune disorders and immunodeficiencies.

The ZSIG-35 polypeptides disclosed herein are contemplated to be useful as therapeutics for treating mammals in need of a ZSIG-35 polypeptide. ZSIG-35 is a member of the chemokine family whose known pro-inflammatory, pyrogenic, chemokinetic, myelosuppressive or hematopoietic effects are being exploited in therapeutic regimes. Depending on the amount, mode of administration and site of administration, chemokines may be made to act in an agonist or antagonist manner. For example, a chemokine may be used in a pro-inflammatory or anti-inflammatory fashion, depending on the mode of administration. ZSIG-35 polypeptides, agonists and antagonists could modulate one or more biological processes in cells, tissues and/or biological fluids.

Expression of ZSIG-35 mRNA in the small intestine and thymus suggests that ZSIG-35 may play a role in lymphocyte development and recruitment. The expression pattern of ZSIG-35 in the thymus may be on epithelial cells and may play a role in recruitment of early thymocyte progenitors to the thymus. It may also play a role in the thymocyte maturation since there is evidence for chemokines having a role in lymphocyte development and activation (Takashi et al., ibid; Hedrick and Zlotnik, Curr. Opin. Immunol. 8:343-47, 1996). Expression of ZSIG-35 in small intestine may be due to inflammation of tissue prior to making RNA used in blot preparation or it may be due to constitutive expression in the small intestine and expression level could increase after injury which suggests a role for ZSIG-35 in intestine injury and inflammatory diseases. The expression of ZSIG-35 in the thymus may be unrelated and the sensitivity of the Northern assay may be too low to detect actual pattern of expression in other cell types. Expression of another chemokine, eotaxin, was highest in small intestine and

colon, but it was also expressed in heart and PCR analysis identified it in spleen, thymus and purified eosinophils as well (Ponath et al., ibid.). Further information on the nature of role of ZSIG-35 in the small intestine could be gained by comparing normal intestinal tissue sections and those taken following inflammation or injury for recruitment of leukocytes to the site of injury.

Localization of ZSIG-35 mRNA expression to the small intestine suggests that ZSIG-35 polypeptides may be beneficial as a treatment for gastrointestinal inflammatory injuries, such as inflammatory bowel disease, colitis and intestinal ulcers and Crohn's disease. These diseases are characterized by an amplification of the inflammatory process, leading to increased numbers of T and B lymphocytes, macrophages, neutrophils and eosinophils found within the mucosa of such patients.

ZSIG-35 polypeptide may have therapeutic use to reduce the damage in ischemic and reperfusion injuries. Such applications would include surgical use, such as for gastrointestinal surgery. In particular, ZSIG-35 may be used to reduce damage to the muscle of the heart associated with coronary occlusion, such as that done during a coronary occlusive heart attack. One factor that influences such injury is neutrophil accumulation in the area.

The chemoattractant properties of ZSIG-35 could be exploited in a wound healing regime to stimulate an infiltration of immune cells (monocytes, neutrophils, T lymphocytes, basophils and the like) to a wound site to facilitate healing. ZSIG-35 may be used to mobilize progenitor cells from the marrow into the peripheral blood for transplants.

ZSIG-35 may play a role in mediating suppression of HIV replication in $CD4^+$ T-cells and limiting progression of HIV infection to AIDS. Other members of the β chemokine family, RANTES, MIP-1α and MIP-1β, and IL-16,

have been shown to be involved in HIV control. Also, ZSIG-35 and its associated receptor could be used to further characterize chemokine-HIV inhibitory activity and for analysis of the pathogenesis of HIV.

ZSIG-35 polypeptides may be useful during chemotherapy or radiation therapy, to protect hematopoietic cells. For instance, chemokine myelosuppressive properties would enhance recovery of progenitor cells following chemo- or radiation therapies, by myelosuppressing normal cells so that only the cancerous cells are sensitive to the therapy.

The invention also provides antagonists, which either bind to ZSIG-35 polypeptides or, alternatively, to a receptor to which ZSIG-35 polypeptides bind, thereby inhibiting or eliminating the function of ZSIG-35. Such ZSIG-35 antagonists would include antibodies, oligonucleotides, natural or synthetic analogs of ZSIG-35 polypeptides, which bind either to the ZSIG-35 polypeptide or to its associated receptor, but do not result in receptor signaling. Such natural or synthetic analogs could be peptides or peptide-like compounds. Natural or synthetic small molecules which bind to receptors of ZSIG-35 polypeptides and prevent signaling are also contemplated as antagonists. As such, ZSIG-35 antagonists would be useful as therapeutics for treating certain disorders where blocking a signal derived from ZSIG-35 would be beneficial.

ZSIG-35 antagonists would have beneficial therapeutic effect in diseases where the inhibition of activation of certain macrophages, neutrophils, basophils, B lymphocytes and/or T cells would be effective. In particular, such diseases would include autoimmune diseases, such as multiple sclerosis, insulin-dependent diabetes and systemic lupus erythematosus. Also, benefit would be derived from using ZSIG-35 antagonists for chronic inflammatory and infective diseases.

Chemokine antagonists are being exploited as therapeutics for treating chronic and acute inflammatory diseases. Bronchial epithelial cells produce chemokines which appear to play a role in local bronchial inflammation, such as asthma and other respiratory distress conditions. Antagonists to chemokines, such as ZSIG-35, could be used as therapeutic treatment for such bronchial inflammation. Chemokine antagonists would also be useful for modulating chemokine levels in the rheumatic joint and to inhibit influx of monocytes into the synovial fluid as a treatment for rheumatoid arthritis. In addition, antagonists may be used to treat some allergies by inhibiting the release of histamine.

ZSIG-35 antagonists would have therapeutic value for treating arteriosclerosis. Arterosclerotic lesions may be macrophage-derived. Use of a chemokine antagonist to block chemokine receptors in arterial smooth muscle cells would reduce the influx of macrophages to arterial walls.

Pharmaceutically effective amounts of ZSIG-35 polypeptides or ZSIG-35 agonists and antagonists of the present invention can be formulated with pharmaceutically acceptable carriers for parenteral, oral, nasal, rectal, topical, transdermal administration or the like, according to conventional methods. Formulations may further include one or more diluents, fillers, emulsifiers, preservatives, buffers, excipients, and the like, and may be provided in such forms as liquids, powders, emulsions, suppositories, liposomes, transdermal patches and tablets, for example. Slow or extended-release delivery systems, including any of a number of biopolymers (biological-based systems), systems employing liposomes, and polymeric delivery systems, can also be utilized with the compositions described herein to provide a continuous or long-term source of the ZSIG-35 polypeptide, agonist or antagonist. Such slow release systems are applicable to formulations,

for example, for oral, topical and parenteral use. The term "pharmaceutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the host or patient. One skilled in the art may formulate the compounds of the present invention in an appropriate manner, and in accordance with accepted practices, such as those disclosed in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995.

As used herein a "pharmaceutically effective amount" of such a ZSIG-35 polypeptide or antagonist is an amount sufficient to induce a desired biological result. The result can be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an effective amount of a ZSIG-35 polypeptide is that which provides either subjective relief of symptoms or an objectively identifiable improvement as noted by the clinician or other qualified observer. In particular, such an effective amount of a ZSIG-35 polypeptide results in reduction in leukocyte recruitment, edema, swelling, reduction in inflammatory cytokines, reduction in pyrogenicity or other beneficial effect. Effective amounts of the ZSIG-35 polypeptides can vary widely depending on the disease or symptom to be treated. The amount of the polypeptide to be administered, and its concentration in the formulations, depends upon the vehicle selected, route of administration, the potency of the particular polypeptide, the clinical condition of the patient, the side effects and the stability of the compound in the formulation. Thus, the clinician will employ the appropriate preparation containing the appropriate concentration in the formulation, as well as the amount of formulation administered, depending upon

clinical experience with the patient in question or with similar patients. Such amounts will depend, in part, on the particular condition to be treated, age, weight, and general health of the patient, and other factors evident to those skilled in the art. Typically a dose will be in the range of 0.1-100 mg/kg of subject.

The dosages of the present compounds used to practice the invention include dosages effective to result in the desired effects. Estimation of appropriate dosages effective for the individual patient is well within the skill of the ordinary prescribing physician or other appropriate health care practitioner. As a guide, the clinician can use conventionally available advice from a source such as the Physician's Desk Reference, 48th Edition, Medical Economics Data Production Co., Montvale, New Jersey (1994).

Preferably the compositions are presented for administration in unit dosage forms. The term "unit dosage form" refers to physically discrete units suitable as unitary dosed for human subjects and animals, each unit containing a predetermined quantity of active material calculated to produce a desired pharmaceutical effect in association with the required pharmaceutical diluent, carrier or vehicle. Examples of unit dosage forms include vials, ampules, tablets, caplets, pills, powders, granules, eyedrops, oral or ocular solutions or suspensions, ocular ointments, and oil-in-water emulsions. Means of preparation, formulation and administration are known to those of skill, see generally Remington's Pharmaceutical Science 15th ed., Mack Publishing Co., Easton, PA (1990).

When necessary, the dosage can be repeated daily, or sometimes twice a day, until either a therapeutic result is achieved or until side effects warrant discontinuation of therapy. Once a therapeutic

effect is achieved, the dosage can be tapered or discontinued.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1
Identification of ZSIG-35

Novel ZSIG-35 polynucleotides and polypeptides of the present invention were initially identified by querying an EST database. To identify the corresponding cDNA, a clone considered likely to contain the entire cDNA sequence was used for sequencing. Using a QIAwell 8 plasmid kit (Qiagen, Inc., Chatsworth, CA) according to manufacturer's instructions, a 5 ml overnight culture in LB broth + 50 µg/ml ampicillin was prepared. The template was sequenced on an Applied Biosystems™ model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.) according the manufacturer's instructions. Oligonucleotides ZC694 (SEQ ID NO:3) and ZC695 (SEQ ID NO:4) to the T7 and SP6 promoters on the vector were used as sequencing primers. Oligonucleotides ZC12777 (SEQ ID NO:5), ZC12779 (SEQ ID NO:6), ZC12782 (SEQ ID NO:7) and ZC12822 (SEQ ID NO:8) were used to complete the sequence from the clone. Sequencing reactions were carried out in a Hybaid OmniGene Temperature Cycling System (National Labnet Co., Woodbridge, NY). SequencherTM 3.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI) was used for data analysis. The resulting 1029 bp sequence is disclosed as SEQ ID NO:1. The sequence of the initially identified EST was confirmed within this sequence.

Example 2
Tissue Distribution

Human Multiple Tissue Northern Blots (MTN I, MTN II, and MTN III; Clontech) were probed to determine the tissue distribution of human ZSIG-35 expression. A 30 bp oligo (ZC12449; SEQ ID NO:9) was used to probe the blots. The 5' end of the probe was radioactively labeled using T4 polynucleotide kinase. To a final volume of 10 µl was added 10 pm ZC12449, 0.1 µl 10X kinase buffer (500 mM Tris-HCl, pH 7.5, 100 mM DTT, 100 mM $MgCl_2$), 0.1 units T4 polynucleotide kinase (GIBCO BRL, Gaithersburg, MD), 20 pm ^{32}P γATP and H_2O. The reaction was incubated at room temperature for 1 hour. The probe was purified using a NUCTRAP push column (Stratagene, La Jolla, CA). ExpressHyb™ (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 42°C using 4 x 10^6 cpm/ml of labeled probe. The blots were then washed at 64°C in 1X SSC, 0.1% SDS. A 1 kb transcript was detected in thymus and small intestine.

Additional Northern analysis was carried out on a gut tissue blot. The blot was prepared using mRNA from human colorectal adenocarcinoma cell line SW480 (Clontech), human small intestine tissue (Clontech), human stomach tissue (Clontech), human intestinal smooth muscle cell line (HISM; ATCC No.CRL-1692; American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD); normal human colon cell line (FHC; ATCC No. CRL-1831; American Type Culture Collection) and human normal fetal small intestine cell line (FHs74 Int.; ATCC No. CCL241; American Type Culture Collection). Total RNAs were isolated from HISM, FHC and FHs74 Int. cells by acid guanidium method (Cheomczynski et al., Anal. Biochem. 162:156-9, 1987). The polyA+ RNAs were selected by eluting total RNA through a column that retains polyA+ RNAs (Aviv et al., Proc. Nat.

Acad. Sci. 69:1408-12, 1972). Two micrograms of polyA+ RNA from each sample was separated out in a 1.5% agarose gel in 2.2 M formaldehyde and phosphate buffer. The RNAs were transferred onto Nytran membrane (Schleicher and Schuell, Keene, NH) in 20X SSC overnight. The blot was treated in the UV Stratalinker 2400 (Stratagene, La Jolla, CA) at 0.12 Joules. The blot was then baked at 80°C for one hour.

A probe was prepared by Eco RI-Not I restriction digest of the clone discussed above in Example 1. The resulting ~1 kb Eco RI-Not I probe was visualized by agarose gel electrophoresis (1.5% agarose gel in 1x TBE), excised from the gel and purified using Qiaex II resin (Qiagen) according to the manufacturer's instructions. The probe contained the full length coding sequence of ZSIG-35 in addition to the 5' and 3' untranslated sequences as disclosed in SEQ ID NO:1. Twenty five nanograms of the probe was radioactively labeled using a MULTIPRIME labeling kit (Amersham) according to the manufacturer's instruction. The reaction was incubated at room temperature for 1 hour. The probe was purified using a NUCTRAP push column (Stratagene, La Jolla, CA). ExpressHyb™ (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 60°C using 2.5 x 10^6 cpm/ml of labeled probe. The blot was washed copiously in 2X SSC, 0.1% SDS at room temperature, then at 65°C in 0.1X SSC, 0.1% SDS, and exposed to film overnight. A 1 kb transcript corresponding to ZSIG-35 was seen in intestine only.

Additional Northern analysis was carried out on a Tumor Panel Blot II (Invitrogen Inc., San Diego, CA) using the probe and hybridization conditions described above for the gut blot. The blot contained RNA from esophageal tumor, normal esophagus, stomach tumor, normal stomach, colon tumor, normal colon, rectal tumor and normal rectum. A transcript of 1.0 kb was detected in

both tumor and normal stomach, colon and rectum tissues. A faint transcript was seen in esophageal tumor tissue but not in normal esophageal tissue.

Additional Northern analysis was also carried out on a Colon Tumor Blot (Invitrogen) using the probe and hybridization conditions described above. The blot contained RNA from colon tumors from four donors, along with normal RNA from the same tissue type. Of the samples from the four donors, strong transcripts corresponding to ZSIG-35 were detected in the tumor tissue of two individuals, in the third, a transcript was detected in both tumor and normal tissue, expression was slightly higher in the normal tissue, and no transcript was detected in tissue from the fourth individual.

Example 3
Chromosomal Assignment and Placement of ZSIG-35.

ZSIG-35 was mapped to chromosome 19 using the commercially available GeneBridge 4 Radiation Hybrid Panel (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains PCRable DNAs from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly available WWW server (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR" radiation hybrid map) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

For the mapping of ZSIG-35 with the GeneBridge 4 RH Panel, 20 μl reactions were set up in a PCRable 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a RoboCycler Gradient 96 thermal cycler (Stratagene). Each of the 95 PCR reactions consisted of 2 μl 10X KlenTaq PCR reaction buffer (CLONTECH Laboratories, Inc., Palo Alto,

CA), 1.6 µl dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 µl sense primer, ZC 13,485, (SEQ ID NO:12), 1 µl antisense primer, ZC 13,490, (SEQ ID NO:13), 2 µl *Redi*Load (Research Genetics, Inc., Huntsville, AL), 0.4 µl 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and ddH_2O for a total volume of 20 µl. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 1 cycle 5 minute denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 60°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The results showed that ZSIG-35 maps 42.17 cR_3000 distal from the top of the human chromosome 19 linkage group on the WICGR radiation hybrid map. Proximal and distal framework markers were RP_S28_1 and WI-8049 (D19S1006), respectively. This positions ZSIG-35 in the 19p13.3 region on the integrated LDB chromosome 19 map (The Genetic Location Database, University of Southhampton, WWW server: http://cedar.genetics.soton.ac.uk/public_html/).

Example 4
Construction of ZSIG-35 Mammalian Expression Vectors ZSIG-35CF/pZP9, ZSIG-35NF/pZP9 and ZSIG-35/pZP9

Three expression vectors were prepared for the ZSIG-35 polypeptide, ZSIG-35CF/pZP9 and ZSIG-35NF/pZP9, wherein the constructs are designed to express a ZSIG-35 polypeptide with a C- or N-terminal FLAG tag (SEQ ID NO:15) and ZSIG-35/pZP9 which expresses the ZSIG-35 polypeptide untagged.

ZSIG-35/pZP9 untagged

A 1037 bp fragment was excised from the clone in Example 1 with restriction enzymes Eco RI and Not I which gives the entire ZSIG-35 sequence including 5' and 3' UTRs as disclosed in SEQ ID NO:1. The excised DNA was subcloned into plasmid pZP9 which had been cut with restriction enzymes Eco RI and Not I. The mammalian expression vector pZP9 has the dihydrofolate reductase gene under control of the SV40 early promoter, SV40 polyadenylation site, a cloning site to insert the gene of interest under control of the MT-1 promoter and the human growth hormone (hGH) polyadenylation site. The expression vector is deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD.

Thirteen nanograms of the Eco RI/Not I digested ZSIG-35 insert and 22 ng of the corresponding vector were ligated overnight at 16°C. Of this ligation reaction, 1.5 µl was electroporated into DH10B competent cells (GIBCO BRL, Gaithersburg, MD) according to manufacturer's direction, plated onto LB plates containing 50 mg/ml ampicillin and incubated overnight at 37 °C. Colonies were screened by PCR using primers ZC6583 (SEQ ID NO:26) and ZC5020 (SEQ ID NO:27) with an initial denaturation of 1 minute 20 seconds followed by 35 cycles at 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 1 minute 30 seconds, ending with a 10 minute extension at 72°C. The insert sequence of positive clones were verified by sequence analysis. Large scale plasmid preparation was done using a QIAGEN® Maxi prep kit (Qiagen) according to manufacturer's instructions.

ZSIG-35CF/pZP9

A PCR generated full length ZSIG-35 DNA fragment was created using a two-part PCR strategy. During construction of this fragment an internal Bam HI site in the coding sequence of ZSIG-35 was also destroyed by incorporating the silent mutation of nucleotide 254 of SEQ

ID NO:1 from C to A maintaining the isoleucine codon (from ATC to ATA). This site was eliminated to allow for convenient cloning into the Bam HI site of the CF/pZP9 vector with the PCR generated ZSIG-35 fragment. To introduce the silent mutation, two separate PCR fragments were generated which would then be joined together by a third PCR reaction. The silent mutation was introduced by oligonucleotides ZC13805 (SEQ ID NO:23) and ZC13806 (SEQ ID: NO:24). For the initial PCR, one reaction contained the oligonucleotide primers ZC13189 (SEQ ID NO:22) and ZC13805 (SEQ ID NO:23) and had the cycle conditions of an initial denaturation at 94°C for 1 minute 30 seconds; followed by 4 cycles at 94°C for 10 minutes, 44°C for 20 seconds and 72°C for 30 seconds, followed by 11 cycles at 94°C for 10 seconds, 64°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. The second reaction contained the primers ZC13806 (SEQ ID NO:24) and ZC13190 (SEQ ID NO:25) and cycle conditions consisting of an initial extension at 94°C for 1 minute 30 seconds; followed by 4 cycles at 92°C for 10 minutes, 44°C for 20 seconds and 72°C for 30 seconds, followed by 11 cycles at 94°C for 10 seconds, 64°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. ZSIG-35/pZP9 from above was used as the template for both reactions and Pfu polymerase and buffer (Stratagene) were used according to the manufacturer's instructions. One twentieth of the resulting PCR reaction products from above were used as a template for a third PCR which joined the two products. Oligonucleotide primers ZC13189 (SEQ ID NO:22) and ZC13190 (SEQ ID NO:25) were used as were the reagents described above. The cycling conditions consisted of an initial extension at 94°C for 1 minute 30 seconds; followed by 12 cycles at 94°C for 10 minutes, 64°C for 20 seconds and 72°C for 45 seconds, ending with a 10 minute extension at 72°C. The resultant PCR product was then purified using a MicroSpin HR S-300 Column

(Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to the manufacturer's instructions. The eluate was then subjected to restriction enzymes Bam HI and Eco RI and the resulting fragment was visualized by agarose gel electrophoresis (1.5% agarose gel with 1X TBE buffer). A band of the predicted size, 456 bp, was excised and the DNA was purified from the gel using Qiaex II resin (Qiagen) according to the manufacturer's instructions.

The excised Eco RI/Bam HI digested DNA was subcloned into plasmid CF/pZP9 which had been cut with Eco RI and Bam HI. The ZSIG-35CF/pZP9 expression vector uses the native ZSIG-35 signal peptide, and expresses a full length ZSIG-35 polypeptide and the FLAG epitope (SEQ ID NO:15) which is attached at the C-terminus of the ZSIG-35 polypeptide as a purification aid. Plasmid CF/pZP9 (deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD) is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, multiple restriction sites for insertion of coding sequences, a sequence encoding the FLAG peptide (SEQ ID NO:15), a stop codon and a human growth hormone terminator. The plasmid also has an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

Twenty five nanograms of the Eco RI/Bam HI digested ZSIG-35 insert and 16 ng of the corresponding vector were ligated overnight at 16°C. Of this, 1.2 microliter was electroporated into DH10B competent cells (GIBCO BRL, Gaithersburg, MD) according to manufacturer's direction and plated onto LB plates containing 50 mg/ml ampicillin, and incubated overnight. Colonies were screened by PCR using primers ZC13006 (SEQ ID NO:28) and ZC13007 (SEQ ID NO:29), reagents as described above, with an initial denaturation of 2 minutes 30 seconds followed

by 20 cycles at 94°C for 15 seconds, 60°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. The insert sequences of positive clones were verified by sequence analysis. Large scale plasmid preparations were done using a QIAGEN® Maxi prep kit (Qiagen) according to manufacturer's instructions.

ZSIG-35NF/pZP9

A full length ZSIG-35 insert was prepared as described above having the addition of a silent mutation to remove a BAM HI restriction site. For the initial PCR, one reaction used the oligonucleotide primers ZC13244 (SEQ ID NO:30) and ZC13805 (SEQ ID NO:23) and had the cycle conditions of an initial denaturation at 94°C for 1 minute 30 seconds; followed by 4 cycles at 94°C for 10 minutes, 44°C for 20 seconds and 72°C for 30 seconds, followed by 11 cycles at 94°C for 10 seconds, 64°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. The second reaction contained the primers ZC13806 (SEQ ID NO:24) and ZC13188 (SEQ ID NO:31) and cycle conditions consisting of an initial extension at 94°C for 1 minute 30 seconds; followed by 4 cycles at 94°C for 10 minutes, 38°C for 20 seconds and 72°C for 30 seconds, followed by 11 cycles at 94°C for 10 seconds, 64°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. ZSIG-35/pZP9 from above was used as the template for both reactions and Pfu polymerase and buffer (Stratagene) were used according to the manufacturer's instructions. One twentieth of the resulting PCR reaction products from above were then used as a template for a third PCR reaction which joined the two PCR products. Oligonucleotide primers ZC13244 (SEQ ID NO:30) and ZC13188 (SEQ ID NO:31) were used as were the reagents described above. The cycling conditions consisted of an initial extension at 94°C for 1 minute 30 seconds; followed by 12 cycles at 94°C for 10 minutes, 64°C for 20 seconds and 72°C

for 45 seconds, ending with a 10 minute extension at 72°C. The resultant PCR product was then purified using a MicroSpin HR S-300 Column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to the manufacturer's instructions. The eluate was then subjected to restriction digestion with Bam HI and Xba I and visualized by agarose gel electrophoresis (1.5% agarose gel with 1X TBE buffer). A band of the predicted size, 414 bp, was excised and the DNA was purified from the gel using Qiaex II resin (Qiagen) according to the manufacturer's instructions.

The excised ZSIG-35 Bam HI/Xba I fragment was subcloned into plasmid NF/pZP9 which had been cut with Bam HI and Xba I. The ZSIG-35/NFpZP9 expression vector incorporates the TPA leader and attaches the FLAG epitope (SEQ ID NO:15) to the N-terminal of the full length ZSIG-35 polypeptide-encoding polynucleotide sequence. Plasmid NF/pZP9 (deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD) is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, a TPA leader peptide followed by the sequence encoding the FLAG peptide (SEQ ID NO:15), multiple restriction sites for insertion of coding sequences, and a human growth hormone terminator. The plasmid also contains an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

Into 16 ng of the NF/pZP9 vector was ligated 8.8 ng of the Bam HI/Xba I ZSIG-35 insert, overnight at 16°C. Of this ligation reaction, 1.5 µl was electroporated into DH10B competent cells (GIBCO BRL, Gaithersburg, MD) according to manufacturer's direction and plated onto LB plates containing 50 mg/ml ampicillin, and incubated overnight. Colonies were screened by PCR

using primers ZC13006 (SEQ ID NO:28) and ZC13007 (SEQ ID NO:29), reagents as described above, with an initial denaturation of 2 minutes 30 seconds followed by 20 cycles at 94°C for 15 seconds, 60°C for 20 seconds and 72°C for 30 seconds, ending with a 10 minute extension at 72°C. The insert sequences of positive clones were verified by sequence analysis. Large scale plasmid preparations were done using a QIAGEN® Maxi prep kit (Qiagen) according to manufacturer's instructions.

Example 5
Expression of ZSIG-35NF/pZP9, ZSIG-35CF/pZP9 and ZSIG-35/pZP9

BHK 570 cells (ATCC No. CRL-10314) were plated in 10 cm tissue culture dishes and allowed to grow to approximately 50 to 70% confluency overnight at 37°C, 5% CO_2, in DMEM/FBS media (DMEM, Gibco/BRL High Glucose, (Gibco BRL, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 1 µM L-glutamine (JRH Biosciences, Lenexa, KS), 1 µM sodium pyruvate (Gibco BRL)). The cells were then transfected with the plasmid ZSIG-35NF/pZP9 (N-terminal FLAG tag), ZSIG-35CF/pZP9 (C-terminal FLAG tag), or ZSIG-35/pZP9 (untagged), using Lipofectamine™ (Gibco BRL), in serum free (SF) media formulation (DMEM, 10 mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). Sixteen micrograms of ZSIG-35NF/pZP9 and 16 µg of ZSIG-35CF/pZP9 were separately diluted into 15 ml tubes to a total final volume of 640 µl SF media. In separate tubes, 35 µl of Lipofectamine™ (Gibco BRL) was mixed with 605 µl of SF medium. The Lipofectamine™ mix was added to the DNA mix and allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media was added to the DNA:Lipofectamine™ mixture. Three plates of cells were rinsed once with 5 ml of SF media, aspirated, and the DNA:Lipofectamine™ mixture was added. The cells were

incubated at 37°C for five hours, then 6.4 ml of DMEM/10% FBS, 1% PSN media was added to each plate. The plates were incubated at 37°C overnight and the DNA:Lipofectamine™ mixture was replaced with fresh FBS/DMEM media the next day. On day 2 post-transfection, the cells were split into the selection media (DMEM/FBS media from above with the addition of 1 μM methotrexate (Sigma Chemical Co., St. Louis, Mo.)) in 150 mm plates at 1:10, 1:20 and 1:50. The cells were refed at day 5 post-transfection with fresh selection media. Approximately 12 days post-transfection, two 150 mm culture dishes of methotrexate resistant colonies from each transfection were trypsinized and the cells were pooled and plated into a T-162 flask and transferred to large scale culture.

Example 6

Large Scale Culture of ZSIG-35 FLAG-tagged polypeptides

A T-162 flask containing confluent cells expressing ZSIG-35/NF was obtained from the expression procedure described above and was expanded into six T-162 flasks. One of the six resulting flasks was used to freeze down four cryovials, and the other five flasks were used to generate a Nunc cell factory.

The cells from the five remaining T-165 flasks were used to seed one Nunc cell factory (10 layers, commercially available from VWR). Briefly, the cells from the T-162 flasks described above expressing ZSIG-35/NF were detached using trypsin, pooled, and added to 1.5 liters ESTEP 1 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L $NaHCO_3$ (Mallinkrodt), 5.0 mg/ml and 25 ml/50L insulin (JRH Biosciences), 10.0 mg/ml and 25 ml/50L transferrin (JRH Biosciences), 2.5L/50L fetal bovine serum (characterized) (Hyclone), 1 μM MTX, with pH adjusted to 7.05 +/-0.05) prewarmed to 37°C. The media containing the cells was then

poured into Nunc cell factories via a funnel. The cell factories were placed in a 37°C/5.0% CO2 incubator.

At 80-100% confluence, a visual contamination test (phenol red color change) was performed on the Nunc cell factories. Since no contamination was observed, supernatant from the confluent factories was poured into a small harvest container, sampled and discarded. The adherent cells were then washed once with 400 ml PBS. To detach the cells from the factories, 100 mls of trypsin was added and removed and the cells were then incubated for 5 to 10 minutes in the residual trypsin. The ZSIG-35NF cells were collected following two, 200 ml washes of ESTEP 1 media. To each of ten ESTEP 1 media-containing bottles (1.5 liters each, at 37°C) was added 40 mls of collected cells. One 1.5 liter bottle was then used to fill one Nunc factory. Each cell factory was placed in a 37°C/5.0% CO_2 incubator.

At 80-90% confluence, a visual contamination test (phenol red color change) was performed on the Nunc cell factories. Since no contamination was observed, supernatant from the confluent factories were poured into a small harvest container, sampled and discarded. Cells were then washed once with 400 ml PBS. Fresh media was added, 1.5 liters of ESTEP 2 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L $NaHCO_3$ (Mallinkrodt), 5.0 mg/ml, 25 ml/50L insulin, 10.0 mg/ml and 25 ml/50L transferrin) was added to each Nunc cell factory. The cell factories were incubated at 37°C/5.0% CO_2.

At approximately 48 hours a visual contamination test (phenol red color change) was performed on the Nunc cell factories. Supernatant from each factory was poured into small harvest containers, 12 liters was collected. Fresh serum-free media (1.5 liters) was poured into each Nunc cell factory, and the factories were incubated at 37°C/5.0% CO_2. One ml of supernatant harvest was

transferred to a microscope slide, and subjected to microscopic analysis for contamination. The contents of the small harvest containers for each factory were pooled and immediately filtered. A second harvest was then performed, substantially as described above at 48 hours, 10 liters obtained, and the cell factories were discarded thereafter. An aseptically assembled filter train apparatus was used for aseptic filtration of the harvest supernatant (conditioned media). Assembly was as follows: tubing was wire-tied to an Opti-Cap filter (Millipore Corp., Bedford, MA) and a Gelman Supercap 50 filter (Gelman Sciences, Ann Arbor, MI). The Supercap 50 filter was also attached to a sterile capped container located in a hood; tubing located upstream of the Millipore Opti-cap filter was inserted into a peristaltic pump; and the free end of the tubing was placed in the large harvest container. The peristaltic pump was run between 200 and 300 rpm, until all of the conditioned media passed through the 0.22 µm final filter into a sterile collection container. The filtrate was placed in a 4°C cold room pending purification.

Concentration and Western Blot

Conditioned media containing ZSIG-35/NF and untagged ZSIG-35 was collected for concentration at various time points (at the 5 T-162 flask stage; 10 factories, fetal bovine serum media; 10 factories, serum free media and a second 10 factory, serum free media time point). Since the expected mass of the protein was in excess of 8 kDA, Millipore 5 kDa cut off concentrators were used. The starting volume for each sample was 15 ml, which was concentrated to a final volume of 1.5 ml. The concentrators were spun at 4°C in Beckman tabletop centrifuge at 2000 x g (3000 rpm) for 40 minutes. The concentrate was transferred to a 1.5 ml non-stick microfuge tube, and the volume was adjusted to 1 ml using

flow through media to achieve a 10x concentration. To sterilize the media, the 10x concentrate was split into two Costar Spin-X tubes, and the tubes were spun at 8000 x g for two minutes in a Eppendorf 5415 microfuge (VWR, Seattle, WA).

Western blot analysis was also conducted for the ZSIG-35/NF samples described above. NuPage gel electrophoresis was conducted using 25 µl of conditioned media and 25 µl 2X reducing sample buffer, according to manufacturer's instructions, running the gel at 150 volts for approximately one hour. The conditioned media sample lanes were loaded with 32.5 µl of sample. Following electrophoresis, the gels were transferred to 2 µm supported nitrocellulose (BioRad) at room temperature for 1 hour (500 mA) using a Hoeffer transfer tank unit (Hoeffer Scientific Instruments, San Francisco, CA) with stirring in accordance with the manufacturer's instructions. The transfer buffer contained 25 mM Tris-Base, 200 mM glycine, and 20% MeOH. Next, the nitrocellulose filters were blocked for 10 minutes at room temperature with 10% non-fat dry milk (NFDM) in Western A buffer (50 mM Tris, pH 7.4; 5 mM EDTA solution, pH 8.0; 0.05% Igepal (Sigma); 150 mM NaCl and 0.25% gelatin). The membrane was then rinsed with Western A buffer. The primary antibody, α-FLAG M2 (Kodak) was added at 0.5 ug/ml in Western A buffer containing 2.5% NFDM with shaking or rocking overnight at 4°C. The membrane was then washed three times for 5 minutes in Western A buffer. A secondary antibody, goat α-mouse IgG-HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added in Western A buffer containing 2.5% NFDM (10 µl of 400 µg/ml antibody solution in 20 mls Western A for a 1:2000 dilution) with shaking or rocking for one hour at room temperature. The membrane was then washed three times for 5 minutes in Western A buffer, and then rinsed in Milli-Q water. The membrane was then placed into a sheet protector (Avery

Office Products, Gold Bar, CA). A 1:1 solution of ECL Western Blotting Detection Reagents (Amersham Life Science., Buckinghamshire, England) was mixed and 500 µl of solution was added to the left edge of the membrane. The solution was then slowly spread over the blot and excess reagent was removed after one minute. The blot was then exposed to ECL Hyperfilm 8x10 (Amersham Corp., Arlington Heights, IL) for 5 seconds.

The results of concentration and Western Blot analysis provided the following concentration and molecular weight estimates:

ZSIG-35/NF:
5 T-162 Flasks = 0.62 mg/L;
10 Factories, FBS = 0.57 mg/L;
10 Factories (#1), SF = 0.62 mg/L; and
10 Factories (#2), SF = 0.21 mg/L.

All samples showed two bands, one a approximately 28 kD and one at approximately 18 kD.

Example 7
Baculovirus Expression Vectors pFSG35 and pFSGE35

Two expression vectors were prepared to express the zsig35 polypeptides in insect cells, pFSG35, designed to express an untagged zsig35 polypeptide and pFSGE35, designed to express a zsig35 polypeptide with a C-terminal Glu-Glu tag (SEQ ID NO:16).

pFSG35

A 473 bp PCR generated zsig35 DNA fragment was created using ZC13415 (SEQ ID NO:17) and ZC13416 (SEQ ID NO:18) as PCR primers and zsig35/pZP9, described above, as a template. The PCR reaction was incubated at 94°C for 2 minutes, followed by 30 cycles of 45 seconds at 94°C, 1 minute at 60°C and 72°C for 1 minute with a 1 second/cycle

segment extension. The resultant PCR product was then run on a 3% gel (2% NuSieve/1% BRL agarose). The 473 bp fragment was captured by diluting 15 fold with 0.1 mM EDTA pH 8.0 and then ligated into the vector pCR2.1 (TA Cloning Kit, Invitrogen Inc., San Diego, CA) according to manufacturer's instructions. The resultant clones were screened for the proper insert orientation and sequenced to confirm identity. The resulting clone was digested with Bgl II and Hind III and the digest run on a 1% SeaPlaque/1% NuSieve agarose gel. A 473 bp band was excised, diluted to 0.5% agarose with 2 mM $MgCl_2$, melted at 65°C and ligated into a Bam HI/Hind III digested baculovirus expression vector, pFastBac1 (Bac-to-Bac™ System, GIBCO-BRL, Gaithersburg, MD). Fifty eight nanograms of the restriction digested ZSIG-35 insert and 196 ng of the corresponding vector were ligated overnight. The ligation mix was diluted 3 fold in TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA).

pFSGE35

A ZSIG-35fragment having a C-terminal Glu-Glu tag was generated by PCR as described above using oligonucleotide primers ZC13415 (SEQ ID NO:17) and ZC13428 (SEQ ID NO:19). A fragment of the expected size, 494 bp, was detected by gel electrophoresis. The DNA fragment was digested with the restriction enzymes Bgl II and Hind III and the resulting 480 bp zsig35 restriction fragment was ligated into a Bam HI/Hind III digested pFastBac1 vector and transformed into DH10α cells as described above, using 200 ng vector and 62 ng ZSIG-35 fragment.

Four fmol of the diluted ligation mixes were independently transformed into DH5α Library Efficiency competent cells (Life Technologies) according to manufacturer's direction by heat shock for 45 seconds in a 42°C waterbath. The ligated DNA was diluted in the appropriate volume of SOC media (2% Bacto Tryptone, 0.5%

Bacto Yeast Extract, 10 ml 1M NaCl, 1.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) and plated onto LB plates containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C. Plasmid DNA was prepared using the QiaVac Miniprep8 system (Qiagen) according the manufacturer's directions. The clones were screened by restriction digest with Hind III/BspE1.

One positive construct was chosen for untagged ZSIG-32 and CEE-tagged ZSIG-35. One microliter of the plasmid DNA from each of these constructs was used to independently transform 20 µl DH10Bac Max Efficiency competent cells (GIBCO-BRL, Gaithersburg, MD) according to manufacturer's instruction, by heat shock at 42°C for 45 seconds. The transformants were then diluted in an appropriate volume of SOC media SOC media and plated on to Luria Agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, IPTG and Bluo Gal. The cells were incubated for 48 hours at 37°C. A color selection was used to identify those cells having virus that had incorporated into the plasmid (referred to as a "bacmid"). Those colonies, which were white in color, were picked for analysis. Bacmid DNA was isolated from positive colonies and screened for the correct insert using PCR. Oligonucleotide primers ZC976 (SEQ ID NO:20) and ZC447 (SEQ ID NO:21) were used and those having the correct insert were used to transfect *Spodoptera frugiperda* (Sf9) cells.

Sf9 cells were seeded at 5 x 10^6 cells per plate and allowed to attach for 1 hour at 27°C. Five microliters of bacmid DNA was diluted with 100 µl Sf-900 II SFM. Six µl of CellFECTIN Reagent (Life Technologies) was diluted with 100 µl Sf-900 II SMF. The bacmid DNA and lipid solutions were gently mixed and incubated 30-45 minutes at room temperature. The media from one plate of cells were aspirated, and the lipid-DNA mixture to which 0.8 ml of Sf-900 II SFM was added. The cells were

incubated at 27°C for 4-5 hours, then 2 ml of Sf-900 II media containing penicillin/streptomycin was added to each plate. The plates were incubated at 27°C, 90% humidity, for 72 hours after which the virus was harvested.

Sf9 cells were grown in 50 ml Sf-900 II SFM in a 50 ml shake flask to an approximate density of 0.04-0.50 x 10^6 cells/ml. They were then transfected with 50 µl of the virus stock from above and incubated at 27°C for 4 days after which time the virus was harvested, and titered, 1.08 x 10^8 pfu/ml. To scale up, five liters of SF 900 II SFM containing SF 9 cells was incubated at 27 °C for and grown for 91 hours. The cells were then transfected with the harvested virus (MOI 0.2) and incubated as above for 71 hours.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: ZymoGenetics, Inc.
1201 Eastlake Ave. E.
Seattle
WA
USA
98102

(ii) TITLE OF THE INVENTION: HUMAN CHEMOKINE ZSIG-35

(iii) NUMBER OF SEQUENCES: 31

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ZymoGenetics
(B) STREET: 1201 Eastlake Ave. E.
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lingenfelter, Susan E.
(B) REGISTRATION NUMBER: 41,156
(C) REFERENCE/DOCKET NUMBER: 97-02PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206) 442-6675
(B) TELEFAX: (206) 442-6678
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1029 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 102...551
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCTTGGCCTA CAGCCCGGCG GGCATCAGCT CCCTTGACCC AGTGGATATC GGTGGCCCCG     60
TTATTCGTCC AGGTGCCCAG GGAGGAGGAC CCGCCTGCAG C ATG AAC CTG TGG CTC    116
                                              Met Asn Leu Trp Leu
                                               1               5

CTG GCC TGC CTG GTG GCC GGC TTC CTG GGA GCC TGG GCC CCC GCT GTC      164
Leu Ala Cys Leu Val Ala Gly Phe Leu Gly Ala Trp Ala Pro Ala Val
               10                  15                  20

CAC ACC CAA GGT GTC TTT GAG GAC TGC TGC CTG GCC TAC CAC TAC CCC      212
His Thr Gln Gly Val Phe Glu Asp Cys Cys Leu Ala Tyr His Tyr Pro
           25                  30                  35

ATT GGG TGG GCT GTG CTC CGG CGC GCC TGG ACT TAC CGG ATC CAG GAG      260
Ile Gly Trp Ala Val Leu Arg Arg Ala Trp Thr Tyr Arg Ile Gln Glu
       40                  45                  50
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GTG AGC GGG AGC TGC AAT CTG CCT GCT GCG ATA TTC TAC CTC CCC AAG      308
Val Ser Gly Ser Cys Asn Leu Pro Ala Ala Ile Phe Tyr Leu Pro Lys
    55                  60                  65

AGA CAC AGG AAG GTG TGT GGG AAC CCC AAA AGC AGG GAG GTG CAG AGA      356
Arg His Arg Lys Val Cys Gly Asn Pro Lys Ser Arg Glu Val Gln Arg
70                  75                  80                  85

GCC ATG AAG CTC CTG GAT GCT CGA AAT AAG GTT TTT GCA AAG CTC CGC      404
Ala Met Lys Leu Leu Asp Ala Arg Asn Lys Val Phe Ala Lys Leu Arg
                90                  95                  100

CAC AAC ACG CAG ACC TTC CAA GCA GGC CCT CAT GCT GTA AAG AAG TTG      452
His Asn Thr Gln Thr Phe Gln Ala Gly Pro His Ala Val Lys Lys Leu
            105                 110                 115

AGT TCT GGA AAC TCC AAG TTA TCA TCG TCC AAG TTT AGC AAT CCC ATC      500
Ser Ser Gly Asn Ser Lys Leu Ser Ser Ser Lys Phe Ser Asn Pro Ile
        120                 125                 130

AGC AGC AGC AAG AGG AAT GTC TCC CTC CTG ATA TCA GCT AAT TCA GGA      548
Ser Ser Ser Lys Arg Asn Val Ser Leu Leu Ile Ser Ala Asn Ser Gly
    135                 140                 145

CTG TGAGCCGGCT CATTTCTGGG CTCCATCGGC ACAGGAGGGG CCGGATCTTT CTCCGAT   608
Leu
150

AAAACCGTCG CCCTACAGAC CCAGCTGTCC CCACGCCTCT GTCTTTTGGG TCAAGTCTTA    668
ATCCCTGCAC CTGAGTTGGT CCTCCCTCTG CACCCCCACC ACCTCCTGCC CGTCTGGCAA    728
CTGGAAAGAG GGAGTTGGCC TGATTTTAAG CCTTTTGCCG CTCCGGGGAC CAGCAGCAAT    788
CCTGGGCAGC CAGTGGCTCT TGTAGAGAAG ACTTAGGATA CCTCTCTCAC TTTCTGTTTC    848
TTGCCGTCCA CCCCGGGCCA TGCCAGTGTG TCCCTCTGGG TCCCTCCAAA ACTCTGGTCA    908
GTTCAAGGAT GCCCCTCCCA GGCTATGCTT TTCTATAACT TTTAAATAAA CCTTGGGGGT    968
TGATGGAGTC AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAG   1028
G                                                                   1029
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 150 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Leu Trp Leu Leu Ala Cys Leu Val Ala Gly Phe Leu Gly Ala
 1               5                  10                  15
Trp Ala Pro Ala Val His Thr Gln Gly Val Phe Glu Asp Cys Cys Leu
            20                  25                  30
Ala Tyr His Tyr Pro Ile Gly Trp Ala Val Leu Arg Arg Ala Trp Thr
        35                  40                  45
Tyr Arg Ile Gln Glu Val Ser Gly Ser Cys Asn Leu Pro Ala Ala Ile
    50                  55                  60
Phe Tyr Leu Pro Lys Arg His Arg Lys Val Cys Gly Asn Pro Lys Ser
65                  70                  75                  80
Arg Glu Val Gln Arg Ala Met Lys Leu Leu Asp Ala Arg Asn Lys Val
                85                  90                  95
Phe Ala Lys Leu Arg His Asn Thr Gln Thr Phe Gln Ala Gly Pro His
            100                 105                 110
Ala Val Lys Lys Leu Ser Ser Gly Asn Ser Lys Leu Ser Ser Ser Lys
        115                 120                 125
Phe Ser Asn Pro Ile Ser Ser Ser Lys Arg Asn Val Ser Leu Leu Ile
    130                 135                 140
Ser Ala Asn Ser Gly Leu
145                 150
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATTTAGGTG ACACTATAG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAAAACCGTT GCCCTACAGA 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGAAGGTCT GCGTGTTGTG 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCCAAGAGA CACAGGAAGG 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGGAGCCCA GAAATGAGCC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGTAGGCCA GGCAGCAGTC CTCAAAGACA 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 148 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Gln Val Ser Val Thr Leu Leu Gly Leu Leu Phe Thr Val Ala Ala
 1               5                  10                  15
Cys Ser Ile His Val Leu Ser Gln Pro Asp Ala Val Asn Ala Pro Leu
            20                  25                  30
Thr Cys Cys Tyr Ser Phe Thr Gly Lys Met Ile Pro Met Ser Arg Leu
        35                  40                  45
```

```
Glu Asn Tyr Lys Arg Ile Thr Ser Ser Arg Cys Pro Lys Glu Ala Val
    50                  55                  60
Val Phe Val Thr Lys Leu Lys Arg Glu Ile Cys Ala Asp Pro Asn Lys
65                  70                  75                  80
Glu Trp Val Gln Lys Tyr Ile Arg Lys Leu Asp Gln Asn Gln Val Arg
                85                  90                  95
Ser Glu Thr Thr Val Phe Tyr Lys Ile Ala Ser Thr Leu Arg Thr Ser
            100                 105                 110
Ala Pro Leu Asn Val Asn Leu Thr His Lys Ser Glu Ala Asn Ala Ser
        115                 120                 125
Thr Leu Phe Ser Thr Thr Thr Ser Ser Thr Ser Val Glu Val Thr Ser
    130                 135                 140
Met Thr Glu Asn
145
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Lys Pro Phe His Thr Ala Leu Ser Phe Leu Ile Leu Thr Thr Ala
 1               5                  10                  15
Leu Gly Ile Trp Ala Gln Ile Thr His Ala Thr Glu Thr Lys Glu Val
            20                  25                  30
Gln Ser Ser Leu Lys Ala Gln Gln Gly Leu Glu Ile Glu Met Phe His
        35                  40                  45
Met Gly Phe Gln Asp Ser Ser Asp Cys Cys Leu Ser Tyr Asn Ser Arg
    50                  55                  60
Ile Gln Cys Ser Arg Phe Ile Gly Tyr Phe Pro Thr Ser Gly Gly Cys
65                  70                  75                  80
Thr Arg Pro Gly Ile Ile Phe Ile Ser Lys Arg Gly Phe Gln Val Cys
                85                  90                  95
Ala Asn Pro Ser Asp Arg Arg Val Gln Arg Cys Ile Glu Arg Leu Glu
            100                 105                 110
```

Lys Asn Ser Gln Pro Arg Thr Tyr Lys Gln
115 120

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGATGCTCGA AATAAGGT 18

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAGGTCTGCG TGTTGTGG 18

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 450 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATGAAYYTNT GGYTNYTNGC NTGYYTNGTN GCNGGNTTYY TNGGNGCNTG GGCNCCNGCN     60
GTNCAYACNC ARGGNGTNTT YGARGAYTGY TGYYTNGCNT AYCAYTAYCC NATHGGNTGG    120
GCNGTNYTNM GNMGNGCNTG GACNTAYMGN ATHCARGARG TNWSNGGNWS NTGYAAYYTN    180
CCNGCNGCNA THTTYTAYYT NCCNAARMGN CAYMGNAARG TNTGYGGNAA YCCNAARWSN    240
MGNGARGTNC ARMGNGCNAT GAARYTNYTN GAYGCNMGNA AYAARGTNTT YGCNAARYTN    300
MGNCAYAAYA CNCARACNTT YCARGCNGGN CCNCAYGCNG TNAARAARYT NWSNWSNGGN    360
AAYWSNAARY TNWSNWSNWS NAARTTYWSN AAYCCNATHW SNWSNWSNAA RMGNAAYGTN    420
WSNYTNYTNA THWSNGCNAA YWSNGGNYTN                                     450
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Asp Tyr Lys Asp Asp Asp Asp Lys
 1               5
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Glu Glu Tyr Met Pro Met Glu
 1               5
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13415

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCCGAGATCT CCATGAACCT GTGGCTCCTG GCCTCGCT 38

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13416

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAGCAAGCTT ACAGTCCTGA ATTAGCTGAT ATCAGGA 37

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13428

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAGCAAGCTT ATTCCATCGG CATGTATTCT TCCAGTCCTG AATTAGCTGA TATCAGGA 58

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC976

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGTTGTAAAA CGACGGCC 18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC447

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TAACAATTTC ACACAGG 17

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13189

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCGGAATTCA TGAACCTGTG GCTC 24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13805

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCACCTCCT GTATCCGATA AG 22

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13806

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTTATCGGAT ACAGGAGGTG AG 22

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13190

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTAGGATCCC AGTCCTGAAT TAGC 24

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC6583

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCCAACGAC TATAAAGAGG G 21

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC5020

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CACTGGAGTG GCAACTTCCA G 21

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13006

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGCTGTCCTC TAAGCGTCAC 20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: 13007

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGGGGTCACA GGGATGCCA 19

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC13244

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ACTGGATCCG CCTGGGCCCC C 21

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAGTCTAGAT TACAGTCCTG AATTAG 26

CLAIMS

What is claimed is:

1. An isolated polypeptide comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2, wherein said polypeptide is a chemokine.

2. An isolated polypeptide according to claim 1, wherein said polypeptide is at least 90% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2.

3. An isolated polypeptide according to claim 1 comprising a sequence of amino acid residues from 16-150 as shown in SEQ ID NO:2.

4. An isolated polypeptide according to claim 1 comprising the sequence of amino acid residues from residue 21-150 as shown in SEQ ID NO:2.

5. An isolated polypeptide according to claim 1 comprising the sequence of amino acid residues from residue 1-150 as shown in SEQ ID NO:2.

6. An isolated polypeptide according to claim 1 further comprising an affinity tag.

7. An isolated polypeptide according to claim 6 wherein said affinity tag is polyhistidine, protein A, glutathione S transferase, substance P, FLAG, Glu-Glu or an immunoglobulin heavy chain constant region.

8. An isolated polypeptide according to claim 7 further comprising a proteolytic cleavage site between said sequence of amino acid residues and said affinity tag.

9. An isolated polynucleotide encoding a polypeptide comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2, wherein said polypeptide is a chemokine.

10. An isolated polynucleotide according to claim 9 wherein said sequence of amino acid residues is at least 90% identical to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2.

11. An isolated polynucleotide according to claim 9, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 16-150 as shown in SEQ ID NO:2.

12. An isolated polynucleotide according to claim 9, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 21-150 as shown in SEQ ID NO:2.

13. An isolated polynucleotide according to claim 9, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 1-150 as shown in SEQ ID NO:2.

14. An isolated polynucleotide according to claim 9 comprising a sequence of nucleotides as shown in SEQ ID NO:14 from nucleotide 1 through nucleotide 450.

15. An isolated polynucleotide according to claim 9 comprising a sequence of nucleotides as shown in SEQ ID NO:1 from nucleotide 162 through nucleotide 551.

16. An isolated polynucleotide according to claim 9 which is from 390 base pairs to 450 base pairs in length.

17. An isolated polynucleotide according to claim 9, wherein said polynucleotide is DNA.

18. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide, said polypeptide comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2, wherein said polypeptide is a chemokine; and

a transcription terminator.

19. An expression vector according to claim 18 further comprising a secretory signal sequence operably linked to said DNA segment.

20. An expression vector according to claim 18 wherein said secretory signal sequence comprises amino acid residues 1-20 of SEQ ID NO:2.

21. An expression vector according to claim 18 wherein said sequence of amino acid residues is at least 90% identical to residues 21 to 150 of SEQ ID NO:2, said polypeptide comprising cysteine residues at positions corresponding to residues 30, 31, 58 and 75 of SEQ ID NO:2.

22. An expression vector according to claim 18, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 16-150 as shown in SEQ ID NO:2.

23. An expression vector according to claim 18, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 21-150 as shown in SEQ ID NO:2.

24. An expression vector according to claim 18, wherein said encoded polypeptide comprises the sequence of amino acid residues from residue 1-150 as shown in SEQ ID NO:2.

25. An expression vector according to claim 18, wherein said DNA segment encodes a polypeptide covalently linked to an affinity tag.

26. An expression vector according to claim 18, wherein said affinity tag is polyhistidine, protein A, glutathione S transferase, substance P, FLAG, Glu-Glu or an immunoglobulin heavy chain constant region.

27. A cultured cell into which has been introduced an expression vector according to claim 18, wherein said cell expresses said polypeptide encoded by said DNA segment.

28. A method of producing a protein comprising:

culturing a cell into which has been introduced an expression vector according to claim 18, whereby said cell expresses said protein encoded by said DNA segment; and

recovering said expressed protein.

29. An antibody that specifically binds to an epitope of a polypeptide according to claim 1.

30. A binding protein that specifically binds to an epitope of a polypeptide according to claim 1.

31. A pharmaceutical composition comprising a polypeptide according to claim 1 and a pharmaceutically acceptable carrier.

32. An oligonucleotide probe or primer comprising 14 contiguous nucleotides of a polynucleotide of SEQ ID NO:14 or a sequence complementary to SEQ ID NO:14.

33. An oligonucleotide probe according to claim 32, wherein said probe is labeled to provide a detectable signal.

34. A method for detecting a genetic abnormality in a patient, comprising:

obtaining a genetic sample from a patient;

incubating the genetic sample with a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product;

comparing said first reaction product to a control reaction product, wherein a difference between said first reaction product and said control reaction product is indicative of a genetic abnormality in the patient.

```
           1         10         20         30         40         50
MCPI_RAT   -MQVSVTLLGLLFTVAACSIHVLSQPDAVNA----------------------P

           1         10         20         30         40         50
MMU49513_1 MKPFHTALSFLILTTALGIWAQITHATETKEVQSSLKAQQGLEIEMFHMGFQDS

           1         10         20
zsig35     MNLWLLACLVAGFLG AWAPA-------------------------VHTQGVF


                60         70         80         90         100
MCPI_RAT   LTCCYSFTGKMIPMSRLENYKRI---TSSRCPKEAVVFVTKLK-REICADPNKE

                60         70              80         90         100
MMU49513_1 SDCCLSYNSR-IQCSRFIGYFP----TSGGCTRPGIIFISKRG-FQVCANPSDR

              30         40         50         60         70         80
zsig35     EDCCLAYHYPIGWAVLRRAWTYRIQEVSGSCNLPAAIFYLPKRHRKVCGNPKSR


              110        120        130        140        150
MCPI_RAT   WVQKYIRKLDQNQVRSETTVFYKIASTLRTSAPLNVNLTHKSEANASTLFST

                    110        120
MMU49513_1 RVQRCIERLEKNSQPRTYKQ

                       90         100        110        120        130
zsig35     EVQRAMKLLDARNKVFAKLRHNTQTFQAGPHAVKKLSSGNSKLSSSKFSNPI


                     170
MCPI_RAT   TTSSTSVEVTSMTEN

                    140        150
zsig35     SSSKRNVSLLISANSGL
```

Fig. 1

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 98/06115

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 C07K16/24 C12N15/62 C12N5/10
A61K38/19 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VICARI A P ET AL: "TECK: A NOVEL CC CHEMOKINE ASSOCIATED WITH T-CELL DEVELOPMENT" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 99, no. 1, PART 02, January 1997, page S246 XP002048195 * see abstract 1003 * ---	1-5, 9-24,27, 28,32,33
X	WO 96 16975 A (DUKE UNIVERSITY) 6 June 1996 see sequence ID no 33 see page 13; claims; table 1 ---	32-34
X	WO 97 08296 A (REGENTS OF THE UNIVERSITY OF MINESOTA) 6 March 1997 see page 49, line 24 - line 26; example 2 --- -/--	32,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Cornec, N

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/06115

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	TOKUYAMA SODA KK.: "A. niger strain M-62 amine oxidase gene degenerate probe" EMBL DATABASE ENTRY T14175, ACCESSION NUMBER T14175, 20 November 1996, XP002072893 see abstract	32,33
X	WO 93 20227 A (ABBOT LAORATORIES) 14 October 1993 see page 14; table 1 see sequnce ID no 27	32,33
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P,X	WO 98 01557 A (SCHERING CORPORATION) 15 January 1998 see the whole document especially sequences ID 3 and 4, the claims see page 49, line 17 - page 50, line 20 see page 61 - page 82	1-34
P,X	VICARI A P ET AL: "TECK: A NOVEL CC CHEMOKINE SPECIFICALLY EXPRESSED BY THYMIC DENDRITIC CELLS AND POTENTIALLY INVOLVED IN T CELL DEVELOPMENT" IMMUNITY, vol. 7, no. 2, August 1997, pages 291-301, XP002048197 see the whole document especially page 298 last paragraph - page 300	1-33
P,A	M.D. ADAMS ET AL: "EST101169 Thymus III Homo sapiens cDNA 5' end" EMBL DATABASE ENTRY HSZZ01097, ACCESSION NUMBER AA295945, 18 April 1997, XP002072465 see abstract	1-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 98/06115

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WO 9616975 A	06-06-1996	AU 4410296 A	19-06-1996
WO 9708296 A	06-03-1997	AU 6855996 A	19-03-1997
WO 9320227 A	14-10-1993	AU 3942993 A	08-11-1993
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